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Flavanol Quantification of Grapes via Multiple Reaction Monitoring Mass Spectrometry. Application to Differentiation among Clones of *Vitis vinifera* L. cv. Rufete Grapes

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Supporting Information

ABSTRACT: The determination of the detailed flavanol composition in food matrices is not a simple task because of the structural similarities of monomers and, consequently, oligomers and polymers. The aim of this study was the development and validation of an HPLC-MS/MS−multiple reaction monitoring (MRM) method that would allow the accurate and precise quantification of catechins, gallocatechins, and oligomeric proanthocyanidins. The high correlation coefficients of the calibration curves (>0.993), the recoveries not statistically different from 100%, the good intra- and interday precisions (<5%), and the LOD and LOQ values, low enough to quantify flavanols in grapes, are good results from the method validation procedure. Its usefulness has also been tested by determining the detailed composition of *Vitis vinifera* L. cv. Rufete grapes. Seventy-two (38 nongalloylated and 34 galloylated) and 53 (24 procyanidins and 29 prodelphinidins) flavanols have been identified and quantified in grape seed and grape skin, respectively. The use of HCA and PCA on the detailed flavanol composition has allowed differentiation among Rufete clones.

KEYWORDS: *quantitative mass spectrometry, HPLC-DAD-MS−multiple reaction monitoring, flavanol, grape seed, grape skin, Vitis vinifera L. cv. Rufete, clonal classification*

INTRODUCTION

Flavanols are monomers, oligomers, and polymers of flavan-3-ol monomers—(epi)catechin and (epi)gallocatechin—which are present in grape seeds and skins and, therefore, in wines and can, in turn, importantly contribute to their mouthfeel properties (mainly astringency and bitterness).[1,2] Differences in sensorial properties have been observed among flavanols of different polymerization degrees. In general, astringency seems to increase as the polymerization degree increases but, on the contrary, bitterness seems to decrease.[2,3] Furthermore, flavan-3-ol units can be esterified with gallic acid and as a result, the intensity of the perceived bitterness and astringency[4] rises. The flavanols of the seeds show higher proportion of galloylated units and smaller molecular weight than those of the skins. Moreover, catechins and procyanidins (flavanols that show only (epi)catechin units in their structure), which are present in both seeds and skins, are related to negative aspects of astringency in contrast to gallocatechins and prodelphinidins (flavanols showing (epi)gallocatechin units in their structure), which can be found only in grape skin and are related to more positive attributes of the astringency sensation.[5] Because of this, it is usually considered that seed flavanols are more undesirable for the elaboration of quality and balanced wines than those from the skin.[6]

Due to the structural similarities among flavan-3-ol monomers and the high number of possible combinations between them that originate oligomers and polymers, the study of the composition of grape flavanols is not simple, and their quantification remains a challenge. Colorimetric methods, usually preceded by flavanol hydrolysis, have typically been used to determine the total content of flavanols.[7−9] However, these methods do not provide information about the profile of flavanols, because they can provide only an estimation of global contents. Furthermore, they are not specific for flavanols, and results can also lack accuracy. Reverse phase chromatography has been the primary method of choice for the detailed analysis of flavanols in foods.[10] Quantification of monomers and oligomers is usually performed from the peaks observed in the chromatogram recorded at 280 nm.[10−12] However, absorption at this wavelength is not exclusive for flavanols and, consequently, quantification from the UV chromatogram may lack accuracy, because it can lead to either an underestimation or an overestimation of the results depending on the baseline and on the integration criteria. Moreover, when the degree of polymerization increases, flavanols (mainly the procyanidins) elute as a single peak at the end of the chromatogram,[13] so this technique does not allow the quantification of the most polymerized oligomers (tetramers, pentamers, etc.).[13,14] Furthermore, unlike in the case of other flavonoids, the UV−vis spectra of flavanols do not provide much useful information for their identification. For this reason, a fractionation of the different flavanols according to their polymerization degree is usually performed prior to the

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analysis,[15] but this can increase the time and cost of the analysis. The coupling of HPLC with mass spectrometry has allowed important progress in the identification of these compounds.[16−18] Although some of them share the same m/z ratios, fragmentation patterns provide useful information to achieve the identification.[19] On the contrary, due to the variability of the mass signal, mass spectrometry has not usually been considered as an alternative technique for quantitative purposes in flavanols determination. Nevertheless, this drawback could be solved by using an appropriate internal standard.

Additionally, there is also a lack of knowledge about the detailed polyphenolic composition of autochthonous and minority grape varieties employed in winemaking. However, nowadays, the interest in these varieties is increasing because wine producers are in search of new types of wines to fulfill wine consumers' demands of *typicity* in opposition to the "standardization" of wines caused by the global use of a few varieties in different growing regions. Moreover, autochthonous varieties seem to be better adapted to the changing conditions of their growing region. Thus, they are less exposed than nonautochthonous varieties to the impact in the quality of the wines made from them as a result of the global warming that has been described to affect wine-growing regions, including European ones.[20] *Vitis vinifera* L. cv. Rufete is autochthonous from a mountainous region called "La Sierra de Francia"[21] that belongs to the Biosphere Reserve "Las Sierras de Béjar y Francia" (Salamanca, Spain) designed by UNESCO in 2006. Rufete wines made in this region, characterized by red fruit notes reminiscent of raspberries and wild strawberries and by spicy and soft tannins, are nowadays recognized with the label "D.O.P. Vino de Calidad de Sierra de Salamanca". However, these wines are less colored than those made from Tempranillo, which is the most cultivated red grape variety in Spain.[21] This lower coloration of the wines is in accordance with the differences observed in the anthocyanin composition between Tempranillo and Rufete grapes.[22] Different strategies can be followed to increase the content of anthocyanins in wine either in the vineyard, by collecting grapes at optimal phenolic maturity, or in the winery, by optimizing the winemaking techniques.[23,24] These strategies are going to condition the levels of other grape constituents such as proanthocyanidins and, consequently, other organoleptic properties of the wines, such as astringency and bitterness. For this reason, anthocyanin and proanthocyanidin compositions have to be well established to select the best strategy to obtain the most colored wine with the most balanced tannins. The anthocyanin and flavonol compositions of Rufete grapes have recently been reported,[22] but nothing is known about the flavanol composition.

The main objective of this work was to develop and validate an HPLC-DAD-MS/MS method that would allow the accurate quantification of grape catechins, gallocatechins, and oligomeric proanthocyanidins. This work also aims to verify the usefulness of the validated method through the study of the detailed flavanol composition of skin and seed of grapes from eight different Rufete clones growing in a reference vineyard belonging to the "D.O.P. Vino de Calidad de Sierra de Salamanca" and to use this composition for chemotaxonomic purposes.

MATERIALS AND METHODS

Chemicals. (+)-Catechin (C), (−)-epicatechin (EC), (+)-gallocatechin (GC), and chlorogenic acid hemihydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Procyanidin dimers B1 and B2, procyanidin trimer C1, (−)-epigallocatechin (EGC), and (−)-epicatechin gallate (ECG) were purchased from Extrasynthèse (Genay, France). All of the used solvents (analytical grade) were purchased from Prolabo (BDH) VWR International (Briare, France). Ultrapure water was obtained from a Direct-Q water purification system equipped with a Millipak 40 (0.22 μm) filter unit (Millipore, Billerica, MA, USA).

Grape Samples. Eight clones of *V. vinifera* L. cv. Rufete red grapes have been analyzed in this study (RF3, RF6, RF20, RF30, RF31, RF32, RF40, and RF48). These clones are the result of a previous clonal selection carried out by the Departamento de Viticultura-ITACYL,[25−27] which selected Rufete vines from "La Sierra de Francia" region on the basis of the results of phenotypic and genetic studies and on the basis of their adequate sanitary status. Then, those vines possessing an adequate sanitary status and phenotypic and genetic features of Rufete variety were all grown in the same vineyard (hereafter, "reference vineyard", located in Villanueva del Conde (Salamanca, Spain, UTM 29TQE58) and belonging to the "D.O.P. Vino de Calidad de Sierra de Salamanca") to reduce the differences that might be induced by different environmental conditions. From all of the clones growing in the reference vineyard, eight were selected for studying their flavanol composition. Samples consisted of 50 grapes collected from different parts of different bunches from the same grapevine. They were collected at technological ripeness (harvest date, September 2, 2014; mean values, 25.05 °Brix; density, 1110 g/L; sugars, 250 g/L; pH, 3.38; total acidity, 5.52 g/L). These 50 grapes were initially weighed. Then, the skins and seeds were manually separated from the whole grape and separately weighed (Table S1 shows the weight of 50 grapes and that of their skins and seeds), thus allowing the determination of the percentage of each part in relation to the whole grape (Table S1). Both skins and seeds were frozen at −20 °C to favor the breakdown of the cells and facilitate the subsequent extraction step.

Phenolic Extracts from Skins and Seeds. The extractions of skins and seeds of each clone were performed in triplicate.

For each skin replicate, 2 g of frozen skins were extracted four times with MeOH/0.5 N HCl (95:5) (30 min with sonication and 12 h of maceration at −20 °C). The extracts were combined, concentrated under vacuum using a rotary evaporator, and redissolved in ultrapure water to a final volume of 20 mL.

Seeds were freeze-dried, and then three aliquots of 0.5 g per clone were crushed and extracted with MeOH/H_2O (75:25) following the procedure proposed by Ferrer-Gallego et al.[28]

HPLC-DAD-MS Analysis. Seed extracts were analyzed after diluting 2.5-fold with ultrapure water. Then, the selected internal standard was added in a final concentration of 0.025 mg/mL.

In the case of skin extracts, they were fractionated prior to the HPLC analysis, with the objective of eliminating the anthocyanins. Fractionation was carried out according to the procedure reported by González-Manzano et al.[29] One milliliter of each extract diluted (1:1) with 0.1 N HCl was loaded on an Oasis MCX cartridge (Waters Corp., Milford, MA, USA) previously conditioned with 2 mL of methanol and 2 mL of water. After a washing with water (4 mL), flavanols and phenolic acids were eluted with 8 mL of methanol. The eluate was concentrated under vacuum and redissolved in 500 mL of water. The selected internal standard was also added prior to the HPLC-DAD-MS analyses to reach the same final concentration as in the seed extracts (0.025 mg/mL).

In both cases, HPLC-DAD analyses were performed in a Hewlett-Packard 1200 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany). The stationary phase was an Agilent Poroshell 120 EC-C18 column (150 × 4.6 mm i.d., 2.7 μm) thermostated at 25 °C. The mobile phase was composed by solvent A, 0.1% (v/v) formic acid aqueous solution, and solvent B, HPLC grade acetonitrile. The following gradient was used at a flow rate of 0.5 mL/min: from 100 to 90% A for 3 min, from 90 to 85.5% A for 34 min, from 85.5 to 80% A for 3 min, from 80 to 65% A for 15 min, from 65 to 40% A for 5 min, and a final isocratic gradient of 40% A for 3 min. Spectra were recorded from 220 to 600 nm, and detection was carried out at 280 nm as the preferred wavelength.

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The mass spectrometer was connected to the HPLC system via the DAD cell outlet. MS detection was performed in a 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple-quadrupole linear ion trap mass analyzer and controlled by Analyst 5.1 software. Nebulizer gas (30 psi) and turbo gas used for solvent drying (300 °C, 40 psi) were zero grade air, whereas nitrogen served as curtain (20 psi) and collision gas (high). Both quadrupoles were set at unit resolution, and the ion spray voltage was set at 5500 V in the positive mode. Multiple reaction monitoring analysis (MRM mode) was employed to detect the transitions (each parent ion−daughter ion pair) corresponding to each kind of flavanol and to the internal standard (chlorogenic acid). Transitions were selected on the basis of the fragmentation pattern of each compound obtained in the analysis of a grape seed extract using the experimental conditions of this study (Table 1). Optimization of the conditions was carried out automatically by direct infusion of catechin, epicatechin, gallocatechin, and epicatechin 3-*O*-gallate. Settings used were as follows: declustering potential (DP), 70 V; and entrance potential (EP), 10 V. Collision energy (CE) was 22, 32, or 40 V depending on the compound and the transition (see Table 1).

Table 1. MRM Transitions Selected for Quantifying Each Group of Flavanols

type of flavanol[a]	parent ion $[M + H]^+$ (m/z)	daughter ions (m/z)	collision energy (V)
(epi)catechin (E)C	291	139	22
PC dimers	579	289 and 291	22
PC trimers	867	577 and 579	22
PC tetramers	1155	577 and 579	32
PC pentamers	1443	577 and 579	32
epicatechin gallate (ECG)	443	139	22
PC monogalloylated dimers	731	289 and/or 291	22
PC monogalloylated trimers	1019	579 and/or 729 and/or 731	32
PC monogalloylated tetramers	1307	577 and/or 579	32
PC monogalloylated pentamers	1595	729 and/or 731	32
PC digalloylated dimers	883	441	22
PC digalloylated trimers	1171	441	32
PC digalloylated tetramers	1459	729	32
(epi)gallocatechin (E)GC	307	139	40
PD dimers	611	305 and/or 307	20
PD mixed dimers	595	289 and/or 305	22
PD mixed trimers	883	593 and/or 579 and/or 577	22, 22, 32
PD double mixed trimers	899	609 and/or 593	22
epigallocatechin gallate (EGCG)	459	139	40

[a]PD, prodelphinidins; PC, procyanidins.

Internal Standard Selection. As a possible internal standard (IS), different compounds that could rarely be found in *V. vinifera* grapes[30] were tested, such as phenolic acids (chlorogenic, ellagic, and rosmarinic acids), flavonols (apigenin 7-*O*-glucoside, kaempferol 7-*O*-neohesperidoside, galangin, and tamarixetin), flavanones (naringenin), flavanonols (taxifolin), anthocyanins (pelargonidin 3-*O*-glucoside), gallotannins (pentagalloylglucose), and isoflavones (daidzin, daidzein, genistin, genistein, and glycitin). A solution containing (+)-catechin, (−)-epicatechin, (+)-gallocatechin, and (−)-epigallocatechin (0.050 mg/mL of each one) was analyzed seven times after the addition of the possible internal standards at the same concentration.

Calibration Curves. (+)-Catechin, (−)-epicatechin, procyanidin dimers B1 and B2, procyanidin trimer C1, (−)-epicatechin 3-*O*-gallate (ECG), (+)-gallocatechin (GC), and (−)-epigallocatechin (EGC) calibration curves were built from MRM quantitative data. Seven levels of concentration in each case were analyzed in triplicate by using the HPLC-MRM method explained above. The ranges of concentrations were (4.5×10^{-5}−0.5 mg/mL) for catechin and epicatechin, (1×10^{-5}−0.1 mg/mL) for gallocatechin and epigallocatechin, and (4.5×10^{-5} and 0.2 mg/mL) for the rest of the external standards. In all cases, chlorogenic acid (0.025 mg/mL) was added as internal standard to all solutions before the analysis. The ratio between the flavanol signal and the internal standard signal (r ratio) was plotted versus the flavanol concentration to build the calibration curves. In the case of catechin, epicatechin, epicatechin 3-*O*-gallate, gallocatechin, and epigallocatechin, the flavanol signal corresponded to the signal of the selected transition, whereas the flavanol signal for procyanidins B1, B2, and C2 was the sum of the signal of the two selected transitions (see Table 1).

Validation of the HPLC-MS Method. To validate the HPLC-MS quantification methods, the following parameters were determined following a procedure previously used for validating mass spectrometry quantitative methods,[31] which is based on the FDA guidance for the validation of analytical methods (see the Supporting Information for further details).

Curve Adjustment. The analysis of the variance of the adjustments was carried out, and the coefficients of correlation were calculated. Moreover, homoscedasticity of variance has been evaluated through Levene's test, showing homogeneity in variance in the linear calibration range.

Accuracy. Accuracy was evaluated through the determination of the recovery rate as follows: (experimental concentration/real concentration) × 100.

Intra- and Interday Precision. Precision was assessed through the coefficients of variation (CV) of the recovery rates determined in the analysis in triplicate of solutions containing catechin, epicatechin, gallocatechin, or epigallocatechin at seven different concentration levels and chlorogenic acid in 1 day (intraday) and on three nonconsecutive days (interday).

Detection (LOD) and Quantification (LOQ) Limits. LOD was determined through the standard deviation (SD) of the r ratio (ratio between the flavanol signal and that corresponding to the internal standard) obtained in the analysis (seven times) of a solution containing the flavanols at a low concentration (providing a signal-to-noise ratio between 2.5 and 5) and the internal standard. LOQ was calculated as 3 times the LOD.

Compound Stability. The stability of the flavanols assayed in the presence and in the absence of the internal standard was tested. In both cases, solutions were analyzed before and after 1 month of storage at 4 °C.

Statistical Analysis. One-way analysis of variance (ANOVA) and Tukey's honestly significant difference test were used to assess the significance of the differences observed among samples. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were used as unsupervised classification techniques to study if the differences among clones allow their separation into different groups. In both cases, total flavanol content and percentages of the main groups of flavanols determined in the present study were used as original variables. In HCA, the similarity matrix was calculated using squared Euclidean distances, and the Ward algorithm was used to generate the dendrogram, whereas PCA was applied from the correlation matrix of the original variables. The IBM-SPSS Statistics 23 for Windows software package (IBM, Armonk, NY, USA) was used to carry out the statistical analysis.

RESULTS AND DISCUSSION

Optimization of the Chromatographic and Mass Spectrometry Conditions. The chromatographic conditions were optimized to allow the best chromatographic separation among the different flavanols. The most important phase of the optimization process consisted of getting a good separation among the galloylated flavanols, which eluted with higher percentages of organic phase (acetonitrile in this method) and that cannot usually be determined because they elute as a single

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peak.[13] Due to this, several tests had to be done to improve the chromatographic resolution in the last part of the chromatogram (last 30 min).

The mass spectrometry method was developed by analyzing seed and skin extracts using a *full mass* method followed by an MS^2 experiment. This allowed the study of the fragmentation patterns of the different flavanols present on grapes. The MRM transitions (each parent ion−daughter ion pair, see Table 1) were selected on the basis of the most important fragments observed in the fragmentation pattern of each flavanol. In the case of monomers (both (epi)catechins and (epi)-gallocatechins, galloylated or not), the most important fragment was found at m/z 139, which corresponds to the fragment originated from the retro-Diels−Alder fission in the C ring of the flavanol.[19] However, in the case of the nongalloylated oligomeric flavanols there was not just a single important fragment but two (or even three in the case of prodelphinidin mixed trimers) fragments showing similar intensities. Moreover, there were no significant differences in the fragmentation pattern among the different oligomers with the same degree of polymerization. With the aim of increasing the sensitivity of the method, both fragments were selected and two transitions were monitored for these compounds, the signal considered for them being the sum of the signal of the two transitions. For instance, in the case of procyanidin dimers, the most intense ion fragments were detected at m/z 291 and 289, which correspond, respectively, to the interflavanic cleavage giving rise to a charged subunit of (epi)catechin and to the loss of a neutral subunit after the quinone methide fission.[19]

On the contrary, in the case of monogalloylated oligomers of procyanidin, the differences in the structure related to the different relative positions of catechin and epicatechin (galloylated or not) in the structure led to differences in the fragmentation patterns. For this reason, there were compounds that were detected at the same m/z but showed different main fragment ions. For instance, in the case of monogalloylated dimers of procyanidin, six peaks showed a *pseudomolecular* ion at m/z 731. Three of them showed only a signal at the 731/289 transition and the other three at the 731/291 transition. Due to this, both transitions were monitored, and each compound was quantified using the signal of the corresponding MRM transition. This fact was also observed in the case of prodelphinidin mixed oligomers due to the differences related to the position of (epi)gallocatechin in the structure, which also led to different fragmentation patterns and, consequently, more than one transition had to be monitored to quantify these compounds (Table 1). Finally, digalloylated flavanols showed a single major fragment ion (m/z 441 for digalloylated dimers and trimers and m/z 729 for digalloylated tetramers), which corresponded to the quinone methide fission of the interflavanic linkage and, consequently, only one MRM transition was used for quantifying these compounds (see Table 1).

Selection of the Most Adequate Internal Standard (IS). In this work, different phenolic compounds have been tested as internal standards to ensure the reproducibility in the quantification of grape flavanols by means of mass spectrometry. From all of the tested compounds only four (the isoflavone glucosides (daidzin, glycitin, and genistin) and chlorogenic acid) did not coelute with other important peaks and showed intermediate retention times. The rest of the compounds were discarded either for eluting at the end of the chromatogram (as occurred in the case of naringenin, taxifolin, and the isoflavone aglycons) or for coeluting with several compounds (as in the case of pelargonidin 3-*O*-glucoside).

A solution containing the four selected compounds and catechin, epicatechin, gallocatechin, and epigallocatechin was analyzed seven times by means of HPLC-MS. The ratio between the signals of each flavanol and each possible IS was determined, and then the coefficient of variation (CV) of this ratio was calculated. The high CV obtained in the case of isoflavone glucosides (>20%) indicated that these compounds were not useful for correcting the variability of the signal of none of the tested flavanols. In the case of chlorogenic acid, the CV of the ratio was <5%, and for this reason this compound was selected as IS. The usefulness of this compound as IS was checked by validating the quantification method.

Method Validation. The developed quantification method was validated through the determination of the calibration curve adjustment, the accuracy, the inter- and intraday precisions, the LOD and LOQ, and the stability of the compounds in the presence and absence of the internal standard.

The correlation coefficients (R^2) were used for assessing the adjustment of the entire calibration curves that had been built (catechin, epicatechin, procyanidin dimers B1 and B2, trimer C1, epicatechin gallate, gallocatechin, and epigallocatechin). All calibration curves showed correlation coefficients >0.993, pointing out the goodness of the correlation. Moreover, the analysis of variance indicated that all of the adjustments were statistically significant ($p < 0.01$).

As explained above, accuracy was assessed through the recovery rates. Due to the broad range of concentrations included in the calibration, accuracy was evaluated at two different levels of concentration. Thus, two solutions containing all of the flavanols employed in building the calibration curves, as well as the IS, were prepared and analyzed in triplicate. The first one was prepared in a lower concentration of flavanols (5×10^{-4} mg/mL of each flavanol in the case of catechins and procyanidins and 2×10^{-4} mg/mL of each flavanol in the case of (epi)gallocatechins), whereas the other contained a higher level of flavanols (6×10^{-2} mg/mL of each flavanol for catechins and procyanidins and 7×10^{-3} mg/mL for (epi)gallocatechins). In both cases, the theoretical concentration and that determined by means of the calibration models were used to calculate the recovery rates. Table 2 showed the values obtained for this parameter, and in all cases they did not show statistically significant differences with a 100% recovery

Table 2. Recovery Rates Calculated for Each Flavanol at the Two Levels of Concentration Assayed

catechins and procyanidins	high concentration[a] (6×10^{-2} mg/mL)	low concentration[a] (5×10^{-4} mg/mL)
(+)-catechin	100 ± 2	99 ± 4
(−)-epicatechin	102 ± 3	101 ± 4
dimer B1	99 ± 2	100 ± 2
dimer B2	101 ± 1	99 ± 3
trimer C1	102 ± 3	100.1 ± 0.7
(−)-epicatechin gallate	101 ± 1	101 ± 3
gallocatechins	high concentration[a] (7×10^{-3} mg/mL)	low concentration[a] (2×10^{-4} mg/mL)
(+)-gallocatechin	100 ± 1	100.4 ± 0.9
(−)-epigallocatechin	101 ± 2	100 ± 1

[a]Interval of confidence calculated at $p = 0.01$ and $n = 3$.

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value, thus indicating that there are no significant differences between the real and experimental concentrations, this latter determined using the calibration model.

The precision of the quantification models was studied both within the same day (intraday) and interday by means of the repetitive analysis of a solution containing catechin and epicatechin (as representative compounds of procyanidins), gallocatechin and epigallocatechin (as representative compounds of prodelphinidins), and the internal standard. The value of the intraday coefficients of variation calculated from the mass signals were 2.0% for catechin, 1.6% for epicatechin, 3.7% for gallocatechin, and 1.2% for epigallocatechin. In the case of interday precision, the values were 3.2, 3.3, 5.1, and 2.8%, respectively. The obtained values were much lower than those proposed by the FDA as upper limits of variability,[32] thus indicating the good precision of the model for flavanol quantification.

The values for the LOD and LOQ are shown in Table 3. The lowest values were obtained for the epigallocatechin model, whereas the model for quantifying galloylated epicatechin showed the highest values of LOD and LOQ. In any case, the values obtained are low enough for quantifying flavanols at the levels observed in grape seeds.[28,33−35]

Table 3. Limit of Detection (LOD) and Limit of Quantification (LOQ) Calculated for Each Quantification Model

compound	LOD (mg/mL)	LOQ (mg/mL)
(+)-catechin	3.53×10^{-6}	1.06×10^{-5}
(−)-epicatechin	1.80×10^{-6}	5.39×10^{-6}
dimer B1	4.39×10^{-6}	1.31×10^{-5}
dimer B2	4.76×10^{-6}	1.43×10^{-5}
trimer C1	9.54×10^{-6}	2.86×10^{-5}
(−)-epicatechin gallate	1.52×10^{-5}	4.57×10^{-5}
(+)-gallocatechin	3.25×10^{-6}	9.75×10^{-6}
(−)-epigallocatechin	9.37×10^{-7}	2.81×10^{-6}

Finally, because chlorogenic acid might affect the stability of tannins (it had been shown to increase the degradation of ellagitannins[31]), the stability of catechin, epicatechin, gallocatechin, and epigallocatechin (as representative compounds of flavanols) was evaluated in the presence and absence of the IS in an aqueous medium. After 1 month of storage at 4 °C, no differences were found between the concentration of each flavanol before and after the storage, neither in the absence nor in the presence of chlorogenic acid. This indicated that, in this case, the IS does not affect flavanol stability, which, in turn, is also stable in a relatively short period of time under storage at 4 °C.

Characterization of Flavanol Composition of Rufete Grapes. The flavanol extracts of the different Rufete clones were analyzed by using the developed and validated HPLC-DAD-MS/MS-MRM method. This methodology allowed the identification and quantification of 72 different catechins and oligomeric procyanidins in the seed extract: 38 nongalloylated (2 monomers, 8 dimers, 9 trimers, 11 tetramers, and 8 pentamers), 28 monogalloylated (1 monomer, 6 dimers, 8 trimers, 8 tetramers, and 5 pentamers), and 6 digalloylated (2 dimers, 3 trimers, and 1 tetramer). In the case of skin extract, it was possible to identify and quantify 53 different flavanols: 24 catechins and procyanidins (2 monomers, 6 dimers, 5 trimers, 6 tetramers, 4 pentamers, and ECG) and 29 gallocatechins and prodelphinidins (2 monomers, 12 dimers, 14 trimers, and EGCG). It is usually considered that the subunits of PCs are catechin and epicatechin, and the subunits of PDs are gallocatechin and epigallocatechin; for this reason, in this work those compounds that showed in their structure both (epi)catechin and (epi)gallocatechin units have been named *mixed* prodelphinidins. Thus, in this work the group named prodelphinidin dimers is really formed by 4 prodelphinidin dimers (M_w 610, i.e., 2 (epi)gallocatechin units) and 8 mixed dimers (M_w 594, i.e., 1 (epi)catechin and 1 (epi)gallocatechin unit) and the group of prodelphinidin trimers is formed by 10 mixed trimers (M_w 882, i.e., 2 (epi)catechin and 1 (epi)-gallocatechin units) and 4 double mixed trimers (M_w 898, i.e., 1 (epi)catechin and 2 (epi)gallocatechin units).

The identity of each compound (Table 1; Tables S2 and S3 in the Supporting Information) was assessed through its chromatographic (retention time) and spectral features (UV spectrum, m/z ratio, fragmentation pattern). In the case of some catechins and procyanidins such as C, EC, and ECG, B-type procyanidin dimers and C1, C2, and EEC (epicatechin-epicatechin-catechin) procyanidin trimers, as well as in the case of gallocatechins (GC, EGC, and EGCG), the identities were confirmed by comparison to the results of analyzing pure solutions of these compounds (purchased or isolated in the laboratory). With regard to prodelphinidins, the identity of some dimers and trimers was assessed on the basis of the fragmentation pattern obtained by mass spectrometry and comparison to those reported in the literature[19] (see Table 1; Tables S2 and S3).

Although the method has allowed the accurate and precise quantification of all the monitored flavanols as individual compounds, due to the large variety of compounds detected in the samples (72 and 53 compounds quantified in seed and skin extracts, respectively) and to simplify the discussion of the results, the compounds were grouped according to the type of proanthocyanidin (procyanindins and prodelphinidins), their polymerization degrees (monomers, dimers, trimers, tetramers, and pentamers), and, in the case of procyanidins, their galloylation degree (nongalloylated, monogalloylated, and digalloylated). In the seed, the mean total content was 29.17 mg of flavanols per gram of seeds (dry weight) with galloylated flavanols accounting for 6.5% of the total content. In the skin of Rufete grapes a mean total content of flavanols was determined as 0.22 mg per gram of skin, being 87.5% procyanidins and 12.5% prodelphinidins (see Tables S4 and S5 for further information about the mean total content and mean percentages of each group of flavanols in the skins and seeds of the Rufete grapes).

When these results are compared to those reported for other grape varieties, it has to be taken into account that the methodologies employed in the different studies were also different. Bearing in mind this consideration, it seems that Rufete seeds possess higher levels of total flavanols than Tempranillo seeds,[36] the most cultivated red grape variety in Spain,[21] but lower levels than in other Spanish varieties such as Graciano.[28] Moreover, dimeric procyanidins are the major group in Rufete seed, whereas in Tempranillo, Graciano, Cabernet Sauvignon, and Tannat seeds, the monomeric flavanols were more abundant than the dimers.[28,33,34] The percentage of galloylation shown by Rufete seeds is similar to that of Tempranillo[34] but lower than those of Graciano[28,34] or Tannat[33] grape seeds. On the contrary, Rufete skins showed much lower levels of flavanols than those reported in

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Table 4. Percentages and Total Content of the Different Groups of Flavanols in the Seeds of the Different Rufete Clones[a]

type of flavanol	RF3	RF6	RF20	RF30	RF31	RF32	RF40	RF46
monomers (%)	30.53 a	29.57 a	25.98 a	27.01 a	33.54 a	31.53 a	29.98 a	31.47 a
dimers (%)	38.16 a	37.98 a	39.8 a	40.41 a	36.34 a	38.25 a	38.07 a	36.07 a
trimers (%)	19.85 a	19.88 a	21.89 a	19.62 a	17.22 a	17.69 a	18.92 a	18.69 a
tetramers (%)	4.43 a	5.02 ab	5.56 ab	5.52 ab	5.92 b	5.28 ab	5.34 ab	5.2 ab
pentamers (%)	0.74 a	0.90 ab	1.04 b	0.98 b	0.96 b	1.02 b	0.99 b	0.95 b
monogalloylated monomers (%)	0.71 bc	0.7 bc	0.48 a	0.76 c	0.73 c	0.84 c	0.52 ab	0.66 abc
monogalloylated dimers (%)	3.59 a	3.80 a	2.75 a	3.14 a	2.85 a	2.92 a	3.93 a	4.49 a
monogalloylated trimers (%)	0.76 a	0.76 a	0.82 a	0.85 a	0.8 a	0.81 a	0.79 a	0.77 a
monogalloylated tetramers (%)	0.79 a	0.90 ab	1.01 ab	1.01 ab	1.01 ab	1.03 ab	0.98 ab	1.15 b
monogalloylated pentamers (%)	0.06 a	0.08 a	0.09 a	0.90 a	0.11 a	0.08 a	0.08 a	0.11 a
digalloylated dimers (%)	0.20 a	0.19 a	0.29 b	0.33 b	0.24 ab	0.32 b	0.19 a	0.18 a
digalloylated trimers (%)	0.17 a	0.21 ab	0.28 b	0.28 b	0.28 b	0.23 ab	0.21 ab	0.25 b
digalloylated tetramers (%)	0.005 a	0.006 a	0.008 ab	0.011 b	0.008 ab	0.008 ab	0.005 a	0.011 b
nongalloylated compounds (%)	93.72 a	93.36 a	94.27 a	93.53 a	93.98 a	93.76 a	93.29 a	92.37 a
galloylated compounds (%)	6.28 a	6.64 a	5.73 a	6.47 a	6.02 a	6.24 a	6.71 a	7.63 a
total content (mg/g seeds, dry wt)	29.17 a	28.96 a	26.85 a	27.30 a	26.86 a	27.90 a	34.00 a	32.32 a

[a]Different lower case letters within each row indicate significant differences ($n = 3$, $p < 0.05$).

Table 5. Percentages and Total Content of the Different Groups of Flavanols in the Skins of the Different Rufete Clones[a]

type of flavanol[b]	RF3	RF6	RF20	RF30	RF31	RF32	RF40	RF46
PC								
monomers (%)	5.7 a	10.4 b	12.3 b	12.5 b	12.0 b	13 b	13.2 b	13.0 b
dimers (%)	36 ab	37 ab	35 ab	37.3 ab	38.7 b	36 ab	37 ab	33.7 a
trimers (%)	31 c	28 abc	27 a	27.6 ab	29.4 abc	30 abc	30.8 bc	31.3 c
tetramers (%)	10 a	8.2 a	8.0 a	8.9 a	8.6 a	9.1 a	8.4 a	10.7 a
pentamers (%)	1.4 a	1.07 a	1.02 a	1.22 a	1.16 a	1 a	1.1 a	1.36 a
ECG (%)	0.28 cd	0.29 d	0.19 abc	0.21 abcd	0.27 bcd	0.16 a	0.18 ab	0.15 a
total PC (%)	85.3 ab	85.6 ab	83 a	87.7 bc	89.9 c	89.4 bc	90.4 c	90.2 c
PD								
monomers (%)	4.3 c	4.6 c	4.9 c	3.36 b	2.56 a	2.8 ab	2.76 ab	2.8 ab
dimers (%)	1.27 a	1.18 a	3.8 a	1.05 a	0.75 a	0.74 a	0.63 a	0.62 a
mixed dimers (%)	4.1 c	4.0 c	3.9 bc	3.897 bc	3.52 abc	3.9 bc	3.17 ab	3.1 a
mixed trimers (%)	2.76 c	2.4 b	2.38 b	2.27 ab	2.10 a	2.1 a	2.05 a	2.25 ab
doubled mixed dimers (%)	1.9 e	1.73 de	1.52 cd	1.38 bc	0.98 a	1.1 ab	1.0 a	1.1 a
EGCG (%)	0.386 d	0.37 cd	0.41 d	0.33 c	0.23 b	0.038 a	0.039 a	0.035 a
total PD (%)	14.7 bc	14.4 bc	17 c	12.3 ab	10.1 a	10.6 ab	9.6 a	9.8 a
total flavanols (mg/g skin)	0.262 d	0.22 abcd	0.240 bcd	0.250 cd	0.211 abc	0.188 a	0.20 ab	0.22 abcd

[a]Different lower case letters within each row indicate significant differences ($n = 3$, $p < 0.05$). [b]PC, catechins and procyanidins; PD, gallocatechins and prodelphinidins; ECG, epicatechin 3-*O*-gallate; EGCG, epigallocatechin 3-*O*-gallate.

Tempranillo,[9,37] Graciano,[11] or Cabernet Sauvignon[38] grape skins, but similar to those reported in Pinot noir[38] or Syrah[9] grape skins. Furthermore, Rufete grape skin showed lower percentages of prodelphinidins than other grape varieties such as Tempranillo,[39] Cabernet Sauvignon, or Merlot.[14]

The detailed flavanol composition of Rufete grapes here reported could be useful for enologists to make decisions during winemaking, because there have been reported several differences in the sensorial properties of flavanols depending on the structure. For instance, higher degrees of galloylation can be related to increased coarseness,[40] and higher polymerization degrees could be associated with a decrease in bitterness and an increase in astringency.[2] Thus, according to this, it seems that the percentage of flavanols of Rufete seeds is quite balanced with respect to astringency and bitterness. Consequently, Rufete grapes could tolerate better than other grape varieties a longer maceration step during winemaking, aiming at extracting higher amounts of pigments. The extraction of flavanols from Rufete seeds during winemaking would increase, in turn, the total flavanol content of the wine, making Rufete grapes compatible with the possibility of making wines for aging, despite the lower flavanol content of the skins in relation to other varieties. Furthermore, the high flavanol content of seeds and their percent composition make Rufete seeds a possible source to be employed as enological tannin.

Statistical Analysis. Tables 4 and 5 show the percent composition and the total concentrations of flavanols in Rufete seeds and skins, respectively. To evaluate the possible differences among different Rufete clones, both HCA and PCA have been performed from the data matrix corresponding to the percentages of the different groups of flavanols and to the total flavanol content in skin or seed. The HCA aimed at determining possible relationships among clones with respect to flavanol composition, whereas PCA was performed to establish the main groups of compounds that were responsible for the separation among clones.

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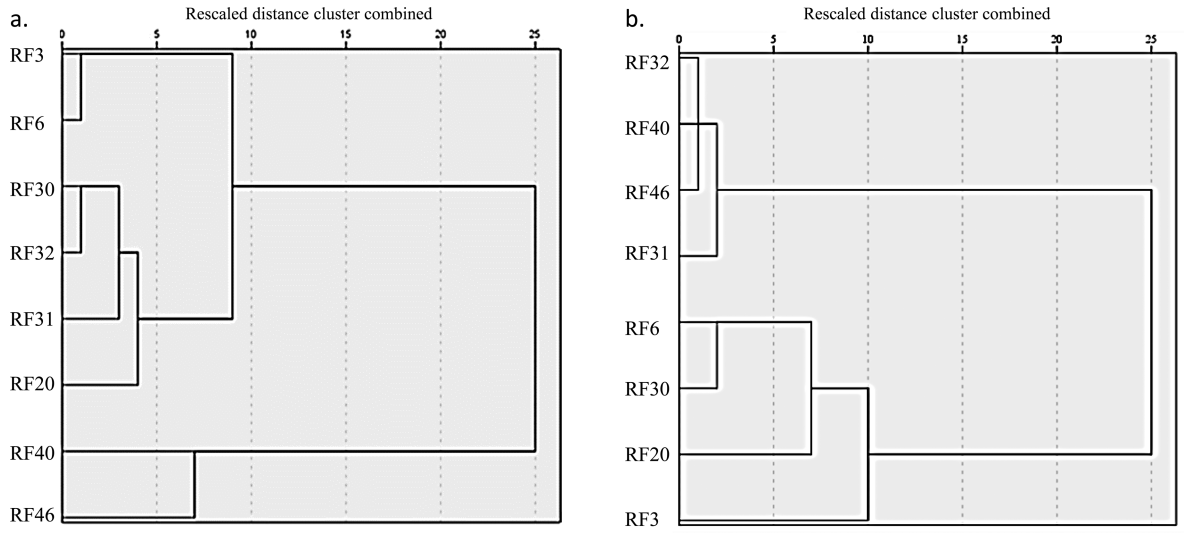

Figure 1. Dendrogram obtained by hierarchical cluster analysis from the seed (a) or skin (b) flavanol content of the different Rufete clones. RF*XX*, different clones of Rufete grapevines.

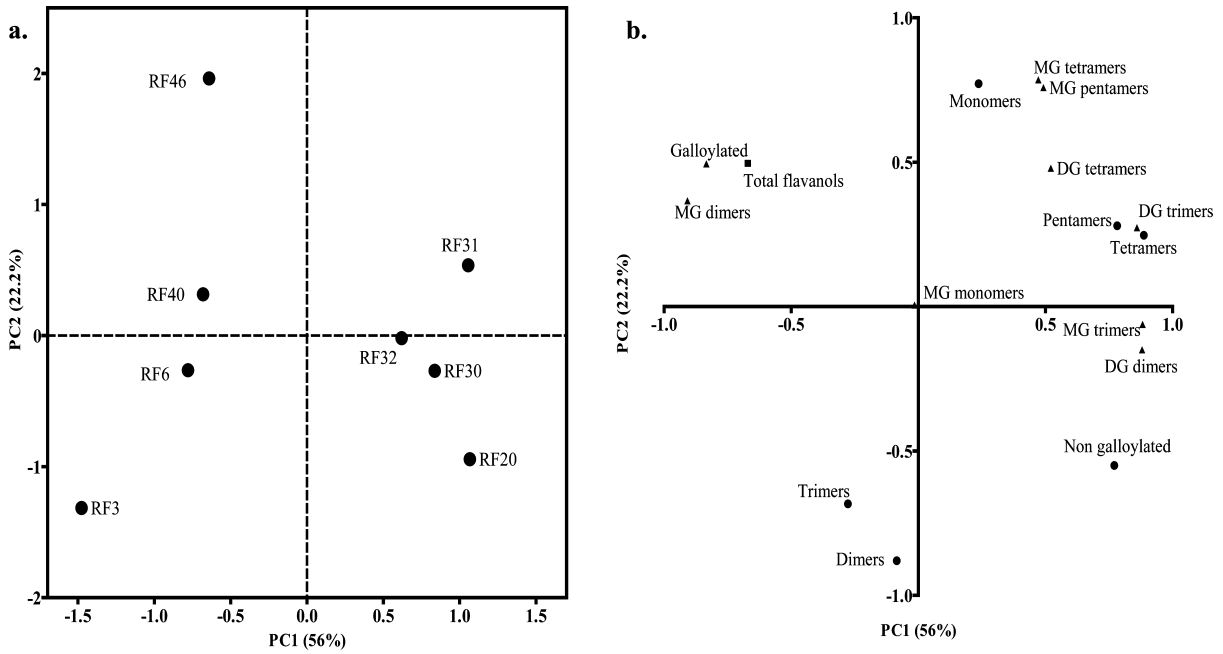

Figure 2. Projection of the samples (a) and loadings of the variables (b) on the two-factor plane resulting from the PCA using the seed flavanol percent composition of the different Rufete clones. MG, monogalloylated; DG, digalloylated.

With respect to HCA, Figure 1 shows the dendrograms obtained in each of the analyses performed. In both cases, clones were first separated into two groups, but these groups were different for seeds and skins. When the data set corresponding to seed composition was used (Figure 1a), one group contained RF40 and RF46 clones and the other group the rest of the clones. This latter was in turn divided in two subclusters: one containing RF3 and RF6, whereas the other contained RF30, RF32, RF31, and RF20 clones. When skin flavanol composition was used as the data set (Figure 1b), one of the initial groups formed, containing RF31, RF32, RF40, and RF46, seemed to be homogeneous. Within the other group only the differentiation of RF3 from the rest of clones from this group (RF6, RF20, and RF30) deserves to be mentioned. Thus, it seems that RF3 is the clone most different from the other clones studied with regard to flavanol composition.

The results of the PCA allowed a deeper study on the types of compounds that contribute more to the separation among clones. Figure 2 shows the distribution of the samples on the plane defined by PC1 and PC2 (a) as well as the loadings (b) of the variables considered for the PCA performed by using the data of the percent composition of the flavanol groups and the total flavanol content of Rufete seeds. The two principal components explain 78.2% of the variance (PC1, 56%; PC2, 22.2%), and a clear separation among samples can be observed. In this case, RF3, RF6, RF40, and RF48 are clearly separated along PC1 from the rest of clones. These four samples showed negative values of PC1, whereas the rest of the samples showed positive values. Negative values are highly correlated with monogalloylated dimers and to total content, whereas positive values are highly correlated with tetramers, monogalloylated trimers, and digalloylated oligomers. This means that, despite

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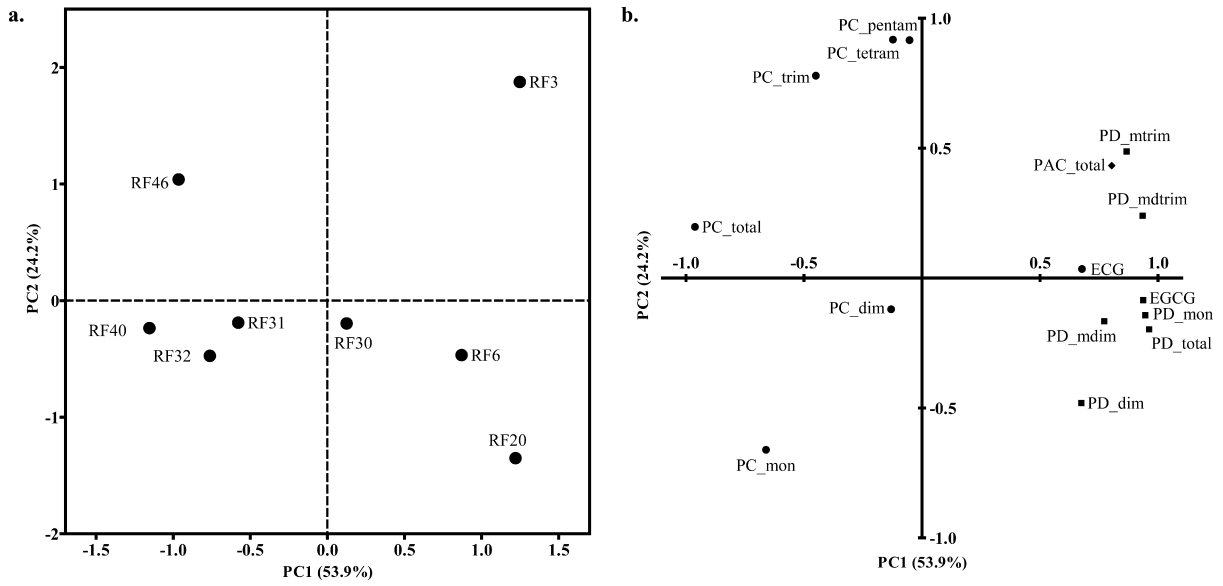

Figure 3. Projection of the samples (a) and loadings of the variables (b) on the two-factor plane resulting from the PCA using the skin flavanol percent composition of the different Rufete clones. PC, procyanidin; PD, prodelphinidin; PAC, proanthocyanidin; mon, monomer; dim, dimer; trim, trimer; tetram, tetramer; pentam, pentamer; mdim, mixed dimer; mtrim, mixed trimer; mdtrim, doubled mixed dimer.

the differences observed in the levels of the different flavanols between RF3−RF6 and RF40−RF46, the proportions of RF3−RF6 are more similar to the latter ones than to the other four samples (RF20, RF30, RF31, and RF32). In terms of astringency, not only the total content of flavanols is relevant but also the proportions of the different groups of compounds.[4] From these results, an increased coarseness might be expected in the case of RF20, RF30, RF31, and RF32, because they correlate with flavanols with higher degrees of galloylation. In general, these four samples showed higher percentages of all the digalloylated oligomers than RF3, RF6, RF40, and RF46, differences that in most cases were statistically significant (Table 4). Samples were further separated along PC2, and this separation is highly and positively correlated with the percentages of monomers, monogalloylated pentamers, and monogalloylated tetramers and negatively correlated with the percentage of dimers and trimers, which can explain the differentiation among RF3 and RF6 from RF40 and RF46.

Figure 3 shows the results of PCA performed by using the data matrix corresponding to the skin composition of flavanols. In this case the separation among groups was similar to that observed in HCA. On the one hand, PC1 explains 53.4% of variability and separates the clones into two groups. RF3, RF6, RF20, and RF30 showed positive values in PC1, whereas RF31, RF32, RF40, and RF46 showed negative values in this PC (Figure 3a). On the basis of the projection of the variables on the plane defined by PC1 and PC2 (Figure 3b), the separation along PC1 can be explained by differences in the percentages of PC or PD. In fact, the clones showing higher positive values in PC1 (RF3, RF6, and RF20) are those that showed significantly higher percentages of prodelphinidins, whereas the higher percentages of procyanidins were found in those clones showing negative values in PC1. RF30 showed a halfway behavior due to the fact that it has intermediate percentages of procyanidins and prodelphinidins. These results are promising because it has been possible to differentiate among clones on the basis of the percentages of procyanidins and prodelphinidins in the skins. Taking into account that prodelphinidins seem to be more related to pleasant oral sensations, whereas procyanidins are more involved in unpleasant ones,[5] studies of this type might be really useful for the complex task of selecting the most adequate clone for elaborating quality wines. On the other hand, PC2 explained 24.2% of variability among clones, allowing separation of RF3 and RF46 clones from the rest of the Rufete clones studied (Figure 3a). This separation can be explained because of the differences in the polymerization degree of the flavanols determined in clones. Thus, clones showing higher percentages of more polymerized flavanols (such as trimers, tetramers, or pentamers, see Table 5) show higher positive values in PC2. This can also be important for selecting the most suitable clone because it has been reported that wines showing higher levels of more polymerized flavanols present a higher intensity of astringency.[40]

In summary, with regard to skin flavanol composition RF3, RF6, and RF20 showed higher percentages of prodelphinidins than the other ones. RF3 was the clone with the lowest percentage of catechins, whereas RF46 and RF40 showed the highest percentages of procyanidins and RF46 also showed higher levels of more polymerized flavanols. Concerning seed flavanol composition, RF3 and RF6 showed the lowest percentages of galloylated compounds and the lowest percentages of tetramers and pentamers (Table 4).

From these results it can be seen that the differentiation of the Rufete clones is possible by the flavanol composition of grape seed and skin. These results are in accordance with those previously reported in which the anthocyanin composition (known as anthocyanin profile) demonstrated its usefulness for chemotaxonomic purposes.[41,42] In fact, a differentiation between Rufete and Tempranillo grapes and between grapes from younger and older Rufete grapevines and even between different clones has been possible through the study of the anthocyanin profile.[22]

The differentiation among clones can be useful because, as previously indicated, one of the drawbacks of the use of the Rufete variety for winemaking is its low coloration in relation to other varieties. Increasing the maceration length time might partly overcome this problem and, in fact, some enologists have used this strategy during the winemaking of Rufete wines.

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However, grape skin has to have an adequate flavanol composition because higher flavanol extraction can be expected with this technique. Furthermore, if the maceration step is extended, the time of contact of the seeds and the wine and, consequently, the time of contact with ethanol, would be also increased, which would provoke a higher extraction of flavanols from the seeds. For this reason, knowledge of the flavanol contents of skins and seeds can be useful for enologists. From the results obtained for the seeds in the present study, the RF3 clone seems to be the best candidate for employing the extended maceration. On the contrary, RF46 and RF40 do not seem to be favorable to the use of this technique if we take into account their flavanol composition of the skin, with low percentages of prodelphinidins. In the case of the other clones with higher total contents or with higher polymerization and/or galloylation degrees in seeds, winemaking techniques such as *delestage* or cold prefermentative maceration would have to be considered to avoid a high extraction of undesired flavanols from the seeds. Nevertheless, further studies focused on the skin composition related to pigments and other phenolic compounds such as flavonols on the same Rufete clones have to be performed to allow the selection of the most suitable clone for elaborating quality wines.

In conclusion, an HPLC-DAD-MS/MS-MRM method has been successfully developed and validated for determining the flavanol composition of grape seeds and skins. This method represents an improvement to the existing methods for the quantification of oligomeric flavanols because it overcomes the coelution problems associated with flavanols due to their structural similarities. The application of this method to the seeds and skins of *V. vinifera* L. cv. Rufete grapes has allowed the precise and accurate quantification of 72 and 53 different flavanols in the seed and in the skin, respectively. The flavanol composition of this grape variety has shown important differences in relation to other varieties usually employed in winemaking, mainly due to the higher total content of seeds, the much lower percentage of galloylation of seed flavanols, and the lower percentages of prodelphinidins in skins. Moreover, the flavanol composition of both seed and skin has been employed to assess the differences among different Rufete clones. The positive results of the present study encourage further research in other wine-grape varieties as part of studies aiming at selecting the clone or clones most suitable for making quality red wines.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b05278.

Validation of the HPLC-MS method; weight of 50 berries and of their corresponding skins and seeds; catechins and procyanidins identified and quantified in the seed extract of Rufete; flavanols identified and quantified in the skin extract of Rufete grapes; mean content and mean percentage of the different groups of flavanols determined in Rufete skins; mean content and mean percentage of the different groups of catechins and procyanidins determined in Rufete seeds (PDF)

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Notes

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